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(54) Title: CELL GROWTH FACTOR RECEPTORS

(57) Abstract

The invention relates to a method of screening a substance for potential utility as a therapeutic agent in the treatment of cancer which comprises providing a standard system in which a protein tyrosine kinase or an active fragment thereof is able to develop a measurable effect, allowing the protein tyrosine kinase to develop that effect in the presence and absence of the said substance and measuring that effect, ability to produce significant inhibition of the effect being taken as an indication of potential utility as a therapeutic agent, wherein the protein tyrosine kinase is characterised by the amino acid sequence of SEQ ID NO. 1 or an amino acid sequence showing a significant degree of homology thereto. The invention also relates to a therapeutic agent thus identified, to a method for detecting the protein tyrosine kinase and an enzyme substrate complex comprising the protein tyrosine kinase and a therapeutic agent capable of modulating the activity of the protein tyrosine kinase.

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CELL GROWTH FACTOR RECEPTORS

The present invention relates to cell growth factor receptors. More particularly the invention relates to the use of a tyrosine kinase growth factor receptor in the development of diagnostic and therapeutic approaches to cancer, for example breast cancer.

Protein tyrosine kinases are enzymes which show the property of catalysing the transfer of phosphate groups from donor molecules (ATP) to the hydroxyl groups of tyrosine residues in polypeptides. Known tyrosine kinases can be classified into two broad groups. Transmembrane tyrosine kinases traverse cellular membranes so that they possess extracellular and intracellular domains. Cytoplasmic tyrosine kinases are located only intracellularly. A general feature of transmembrane (also referred to as receptor) tyrosine kinases is that they possess extracellular ligand-binding domains, hydrophobic transmembrane sequences, and intracellular portions which include the tyrosine kinase domains (for a review see Ullrich & Schlessinger Cell, 61, 203-212 (1990)).

Tyrosine kinases can induce cell proliferation, cell transformation and regulate developmental events (see reviews by Hanks *et al*, Science, 241, 42-75 (1988) and Cantley *et al*, Cell 64, 281-302 (1991)). In general, where functional assays have been available (e.g. mitogenesis or transformation), it has been shown that the biological functions of tyrosine kinases are usually dependent on intact enzyme activity and that through autophosphorylation and the phosphorylation of other proteins, they alter the subcellular localisation and activities of various components of the intracellular signalling pathways.

Analysis of the oncogenes of many acutely transforming animal retroviruses has revealed that their products frequently manifest tyrosine kinase activity, as do the

products of their cellular proto-oncogene counterparts. Other genes encoding tyrosine kinases have been found to be altered by DNA rearrangements in cancer cells, the result being a presumed acquisition of cellular transforming activity (for example c-abl, c-met). Some cellular proto-oncogenes encoding tyrosine kinases have been cloned independently by virtue of the fact that they encode growth factor receptors, for example the epidermal growth factor receptor. On the other hand, the genes for other growth factor receptors which have tyrosine kinase activities, such as those for platelet-derived growth factor and insulin-like growth factors, have been well characterised, but have never been found to be transduced by retroviruses.

The c-erbB-2/HER2/c-neu gene encodes a transmembrane receptor-like tyrosine kinase which is structurally very similar to the EGF/TGF alpha receptor. The c-erbB-2 gene has been found to be overexpressed in 20 to 30% of human breast tumours, often in association with gene amplification, and this phenotype is now generally accepted as predictive of poor disease free and overall survival (for reviews see Sunderland & McGuire in Regulatory Mechanisms in Breast Cancer, Lippman & Dickson (Eds.), Kluwer Academic Publishers, Boston, pages 3 to 22 (1991) and Gusterson *et al.*, J. Clin. Oncol., 10, 1049-1056 (1992)). Overexpression of the gene in fibroblasts induces transformation (di Fiore *et al*, Science, 237, 178-182 (1987) and Hudziak *et al*, Proc. Natl. Acad. Sci. (USA), 84, 7159-7162 (1987)), and ligands which bind to the c-erbB-2 gene product (human and rodent) and activate its tyrosine kinase activity have been identified (Lupu *et al*, Science, 249, 1552-1555 (1990), Dobashi *et al*, Proc. Natl. Acad, Sci. (USA), 88, 8582-8586 (1991), Wen *et al*, Cell, 69, 559-572 (1992) and Holmes *et al*, Science 256, 1205-1210 (1992)). Some of these ligands increase the rate of proliferation of cells expressing the c-erbB-2 protein. The strong implication of these and other findings is that overexpression of this tyrosine kinase in some breast tumour cells is one of the important steps in their progression towards tumourigenicity, and therefore that c-erbB-2 can function as an oncogene.

A variety of other receptor tyrosine kinases, including those for the IGFs and the FGFs, are expressed in breast tumours (Stewart *et al*, J. Biol. Chem., 265 21172-21178 (1990) and Wellstein & Lippman in Molecular Foundations of Oncology, Broder (Ed.), Williams and Wilkins, Baltimore, pages 403-418 (1991)), and the EGF/TGF alpha receptor is overexpressed in some cases (for example Horak *et al*, Oncogene, 6, 2277-2284 (1991)). As with c-erbB-2, overexpression of EGFs has been correlated with poor prognosis. Since the ligands of some of these receptors can be detected in breast tumour samples, it may be that breast tumour development is also regulated by these molecules (for a review see van de Vijver & Nusse, Biochim, Biophys. Acta, 1072, 35-50 (1991)).

The features which are ideally required in a molecular target for tumour therapies include preferential expression in the tumours, a role in the development of the tumours (rather than merely being a marker), and a knowledge of the mechanism of its action as a starting point for the rational design of activity modulators. It appears that all of these three criteria are satisfied, at least to some extent, in the case of c-erbB-2 and breast cancer and a considerable research effort is currently being directed to this molecule.

It has recently been found that the alkaloid K252a, at appropriate concentrations, selectively inhibits the tyrosine kinase and biological activities of the trk-class of neurotrophin receptors, but has no effect on the activities of v-src, v-fms or the receptors for EGF and PDGF, and also does not affect general cell viability or proliferation (Tapley *et al*, Oncogene, 7, 371-381 (1992)). Members of another class of molecules, the tyrphostins, may be selective inhibitors of the EGFr/c-erbB-2 class of tyrosine kinases (Gazit *et al*, J. Med. Chem., 34, 1896-1907 (1991)). An antibody which inhibits the proliferation of human tumour cells by binding to the c-erbB-2 protein is currently under evaluation as a potential cancer therapy (Carter *et al*, Proc. Natl. Acad. Sci. (USA), 89, 4285-4289 (1992)).

If the transformation of a significant minority of breast tumour cells is due, at least in part, to the disregulated activity of the c-erbB-2 tyrosine kinase, then it seems likely that other breast tumours owe aspects of their transformed phenotype to the activity of other tyrosine kinases. The identification of further tumour associated tyrosine kinases would allow the development of novel diagnostic and therapeutic approaches to cancer and in particular breast tumours.

The cDNA cloning of a previously novel receptor tyrosine kinase from breast carcinoma cells has recently been reported (Johnson *et al.*, Proc. Natl. Acad. Sci. USA, 90, 5679-5681 (June 1993)) and the DNA and predicted amino acid sequence of this protein are set out in

SEQ ID NO. 1.

The predicted amino acid sequence is set out separately in

SEQ ID NO. 2.

The authors designated this protein tyrosine kinase DDR (Discoidin domain receptor) and detected the transcription product of the DNA sequence in other human breast carcinoma cell lines and the translation product (protein) was also detected in breast carcinoma cell lines. However, the authors do not suggest that the protein tyrosine kinase plays any role as a tumour causative agent and no potential utility is suggested in the therapy or diagnosis of cancer, in particular human breast cancer.

Another recent disclosure suggests that the same protein tyrosine kinase has been detected in an epithelial ovarian cancer cell line (Laval *et al*, American Association for Cancer Research, Meeting, May 1993, Abstract No. 3144).

It has now been found that the receptor tyrosine kinase referred to above is expressed at a much higher level in human breast tumour cells than in normal breast. This suggests an

involvement of the tyrosine kinase in the transformation of breast cells. The present invention relates to the use of the tyrosine kinase as defined above in the diagnosis and/or therapy of cancer, particularly human breast tumours.

The present invention involves the use of a DNA isolate encoding a protein tyrosine kinase having the amino acid sequence shown in

SEQ ID NO. 1

or an amino acid sequence showing a significant degree of homology thereto, preferably at least 60% homology in the catalytic domain and at least 40% homology in the remainder of the molecule, more preferably at least 90% homology overall, for example at least 95% homology, or a fragment thereof.

According to one embodiment, the DNA isolate encodes all of the amino acid sequence shown in the SEQ ID NO. 1 or an amino acid sequence showing a significant degree of homology thereto. According to another embodiment the DNA isolate encodes a fragment of the amino acid sequence of the protein tyrosine kinase or an amino acid sequence showing a significant degree of homology thereto. DNA sequence encoding fragments of the protein tyrosine kinase preferably encodes those parts of the amino acid sequence which characterise the enzyme, i.e. those parts which are most distinct from other protein tyrosine kinases. Most preferably the DNA sequence encodes all or part of the catalytic domain of the enzyme, i.e. amino acid residues 617 to 907 as shown in SEQ ID NO. 1.

The DNA isolate may have the base sequence defined in SEQ ID NO. 1 to encode the relevant amino acid sequence. Alternatively the DNA isolate may have any other DNA sequence encoding the relevant amino acid sequence.

The DNA isolate may take the form of a cloning vector or an expression vector preferably a plasmid vector, including DNA as defined above. In the case of an expression vector the DNA will be under control of an appropriate promoter and will

include regulatory elements required for expression in a suitable host cell.

The present invention also involves the use of a recombinant cell line transformed with an expression vector as defined above and capable of expressing a recombinant protein tyrosine kinase having amino acid sequence shown in SEQ ID NO. 1 or an amino acid sequence showing a significant degree of homology thereto or a fragment thereof.

Suitable host cells include mammalian cells, insect cells, yeast cells and bacterial cells and the expression vector will include a promoter and other regulatory elements appropriate to the host cell in question. Preferred host cells include CHO cells, myeloma cells, primary immortalised human breast cells, rodent fibroblast cell lines, baculovirus cells, yeast and *E. coli* cells. General techniques for manipulating DNA coding sequences and expressing such sequences in various types of cells are well known to those skilled in the art and are described for example in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory.

The present invention also involves the use of a protein tyrosine kinase having the amino acid sequence shown in SEQ ID NO. 1 or an amino acid sequence showing a significant degree of homology thereto or a fragment thereof.

The protein tyrosine kinase or a fragment thereof may be a recombinant polypeptide produced as the expression product of a coding sequence as defined above in a recombinant cell line. Alternatively the protein tyrosine kinase may be produced in a human tumour cell line. In either case the protein may be extracted and purified by standard techniques, for example antibody affinity chromatography. The glycosylation of the protein (if any) will depend on the cells in which it is produced and protein produced in human tumour

cells in culture should have glycosylation equivalent to that produced *in vivo* in human tumours.

Part of the DNA sequence encoding the protein tyrosine kinase set out in SEQ ID NO. 1 was isolated in the manner described in more detail in the example set out below but which involves briefly the following steps:

i) isolation of mRNA from tumour metastatic tissue;
ii) preparation of cDNA from the isolated mRNA;
iii) PCR amplification using degenerate oligonucleotide primers designed to amplify sequence associated with protein tyrosine kinases;
iv) subcloning PCR products;
v) identification of protein tyrosine kinase products amplified at higher levels from tumour bearing tissue;
vi) DNA sequencing and identification of novel protein tyrosine kinase sequence;
vii) isolation of cDNA sequence 3′ of the region amplified by 3′ RACE PCR.

Application of the above protocol identified part of the DNA sequence for the protein tyrosine kinase which was designated PTK22 and which corresponds to the protein tyrosine kinase designated DDR by Johnson *et al* (Supra). As already noted, the enzyme has now been shown to be overexpressed in certain human breast tumours as compared to normal breast tissue.

The present invention thus relates to the application of the tyrosine kinases for the development of therapeutic, prognostic and diagnostic approaches to cancer. The invention is particularly applicable to breast cancer, however, the same approaches may also be applicable to other cancers and research data suggests a connection between breast cancer and ovarian cancer and also between these two cancers and gastric cancer.

In terms of therapy, the involvement of the tyrosine kinase in tumours means that beneficial clinical effects in

the treatment of tumours can be obtained by modulating the tyrosine kinase activity and/or the functionality of the receptor. There are a number of ways in which such modulation could be achieved.

The activity of the protein tyrosine kinase could be significantly impaired or inhibited by small chemical molecules and screens to identify suitable small molecular weight inhibitors can be developed as described in more detail below. This approach is analogous to that proposed for other tyrosine kinases (see "Drugs of the Future", 17(2), 119-131, (1992)). A further embodiment of this approach would be to derive nmr spectra or crystal structures for the protein tyrosine kinases or domains thereof and use the structural information so obtained to synthesise chemical structures de novo which could be similarly screened for activity as inhibitors.

The approach described above could be extended to encompass small peptides which are either competitive for signalling action of the tyrosine kinase receptor, or which demonstrate useful binding thereto, such that the receptor function is inhibited. This would include, for example, peptides which are capable of blocking substrate binding to the receptor, including but not limited to those which bear SH2 domains. An additional approach is to use small molecules or peptides to block or interfere with ligand activation or dimerisation of the tyrosine kinase. Both of these events are generally required to effect a biological signal so that blocking or inhibition thereof could be used to therapeutic effect.

Macromolecules, such as antibodies, could also be used as inhibitory molecules to block receptor function. This would be particularly applicable to antibodies raised against the extracellular domain of the protein tyrosine kinase where antibodies could promote internalisation or interfere with ligand binding. Either of these situations could produce significant modulation (positive or negative) of the activity

of the protein.

Protein tyrosine kinase function could also be blocked by expressing or administering a peptide which is a truncated or altered version of the protein. These are typically referred to as dominant negative proteins and are believed to sequester the active protein in a non-functional complex. Protein tyrosine kinase function could also be modulated by blocking translation of the mRNA encoding the protein using antisense oligonucleotides.

A further regulatory role of the protein tyrosine kinase could be manipulated by controlling expression or activity of specific phosphatases which control the degree of protein phosphorylation. It is recognised that the phosphorylation of specific residues, particularly tyrosine, threonine and serine residues, play an important role in transmitting a biological signal from the tyrosine kinase.

Use of the above therapeutic approaches depends on the identification and/or development of suitable agents for modulating, for example inhibiting, particular functions or activities of the protein tyrosine kinase. The present invention thus relates to assays or screens by which such agents can be identified.

The present invention provides a method of screening a substance for potential utility as a therapeutic agent in the treatment of cancer, in particular breast cancer, which comprises providing a standard system in which a protein tyrosine kinase or an active fragment thereof is able to develop a measurable effect, allowing the protein tyrosine kinase to develop that effect in the presence and absence of the said substance and measuring that effect, ability to produce significant inhibition of the effect being taken as an indication of potential utility as a therapeutic agent, wherein the protein tyrosine kinase is characterised by the amino acid sequence shown in

SEQ ID NO. 1

or an amino acid sequence showing a significant degree of homology thereto.

In accordance with this general principle, screens for potential therapeutic agents can take a number of forms. For example an initial screen to determine whether or not a substance merits further investigation as a potential inhibitor of the protein tyrosine kinase will usually be biochemical and should preferably be simple, rapid and capable of high through-put. Such screens will often make use of the protein expressed in a recombinant expression system or derived from a cellular source in which the protein is overexpressed. Particularly suitable recombinant expression systems include insect baculovirus, and the use of primary immortalised human breast cell lines, or rodent fibroblast cell lines as host cells. The protein is then used directly in an *in vitro* assay with and without the potential inhibitor. This gives rapid data on the performance of the substance as an inhibitor often providing direct evidence that the substance can inhibit the enzymic activity of the protein tyrosine kinase and thus merits further study. This type of isolated system can also provide data on specificity but will not generally provide any information on bioavailability. The enzymic activity of the protein tyrosine kinase in vitro can be measured, for example, by measuring autophosphorylation or phosphorylation of a model substrate, by following incorporation of radioactive phosphate or by using anti-phosphotyrosine antibodies.

Further information concerning potential inhibitors can be provided by cell based screens which make use of a phenotypic alteration, e.g. a change in morphology and/or tumorigenicity, conferred by expression of the protein tyrosine kinase in a recombinant cell line or overexpression in any other available cell line. Particularly suitable recombinant cell lines again use primary immortalised human breast cell lines or rodent fibroblast cell lines as host cells. The cells can be used in proliferation or tumorigenicity assays with and without the potential inhibitor

looking for substances able to halt growth of and/or morphologically de-transform the cells. A control for substances which are generally cytotoxic can be provided by the same parental cell line engineered to express another oncogene which transforms the cells at a signalling point downstream of the protein tyrosine kinase. Assays of this sort can be very informative and they may provide data on mode of action of inhibitors.

The final stage of screening is the development of animal screens. These are time consuming and expensive and for these and ethical reasons their use is kept to a minimum, however they can give vital information regarding metabolism, clearance and performance of a substance in a true *in vivo* system which cannot be obtained in other ways. Animal screens ideally use the same recombinant or other cell lines as described above which express the protein tyrosine kinase. The cells are used as xenografts in animals, for example, nude mice, to give tumours whose growth is controlled by expression of the protein tyrosine kinase. The mice are then given inhibitors to determine whether they are capable of causing the selective regression of the xenografts without blocking the growth of a control xenograft. Alternatively transgenic animals which develop tumours by virtue of the appropriate tissue specific expression of the target protein tyrosine kinase can be used in a similar manner.

The invention also extends to therapeutic agents identified by use of any or all of the screens referred to above. Preferably the therapeutic agent is a chemical molecule of relatively low molecular weight, for example, less than about 1000. Examples of suitable classes of molecule include staurosporine analogues, tyrphostins and flavenoids. Alternatively, the therapeutic agent can be a macromolecule, for example an antibody raised against the extracellular domain of the protein tyrosine kinase. The therapeutic agent can also be a peptide or an antisense oligonucleotide capable of blocking translation of the mRNA encoding the protein tyrosine kinase.

The invention also extends to an enzyme-substrate complex which comprises a protein tyrosine kinase characterised by the amino acid sequence shown in

SEQ ID NO. 1

or an amino acid sequence showing a significant degree of homology thereto and a therapeutic agent capable of modulating the activity of the said protein tyrosine kinase.

The protein tyrosine kinases can also be used in prognostic and diagnostic applications. Thus antibodies raised against the protein tyrosine kinase or nucleic acid encoding the tyrosine kinase can be used as the basis for screening tissue, in particular tumour tissue, for the presence of the protein tyrosine kinases. If, as in the case of c-erbB-2, the expression of the tyrosine kinase correlates with poor prognosis, then treatment of the patient could be modified accordingly. Similarly, again as with c-erbB-2, there may be situations where parts of the receptor are "shed" into the bloodstream. In this case, the presence of the receptor could be detected, for example, by use of an ELISA assay, and this would be a useful diagnostic tool for the identification of patients predisposed to develop or liable to develop tumours, in particular breast cancer.

Accordingly the present invention provides a method for detecting a tyrosine kinase having an amino acid sequence as defined in

SEQ ID NO. 1

or a fragment thereof which comprises reacting a test sample with a specific antibody raised against an antigen from the said amino acid sequence and determining whether there is any antigen-antibody binding ,within the test sample The test sample may be for example a tissue sample, such as a tumour sample, or a blood sample and presence of abnormal amounts of the protein tyrosine kinase may indicate development of or susceptibility to develop a tumour.

The invention also provides a method for the detection in a sample of DNA a nucleic acid sequence encoding a tyrosine

kinase which comprises subjecting the sample or nucleic acid isolated therefrom to a method capable of detecting a nucleic acid sequence therein identical or substantially homologous to a defined nucleic acid sequence and identifying any sequence identical or substantially homologous to all or part of the DNA sequence defined in

SEQ ID NO. 1,

or mRNA which would be the transcription product of such DNA. Again, presence of abnormal amounts of nucleic acid encoding the protein tyrosine kinase may indicate development of or susceptibility to develop a tumour.

Suitable methods for the detection of specific DNA sequences include Southern blotting and/or PCR using appropriate primers. Suitable methods for detecting mRNA include Northern blotting, RNAse protection studies and direct nucleic acid *in situ* hybridisation.

EXAMPLE

Experimental work on which the invention is based is described in more detail in this Example in which reference is made to the accompanying drawings in which:

Figure 1 shows a Northern blot to examine expression of DDR mRNA in human axillary lymph nodes. 5μg of total RNA were loaded per lane. Approximately 4 kb DDR transcript (upper panel). Ethidium bromide staining of 28S rRNA, to demonstrate RNA loading, is shown (bottom panel).

Figure 2 shows *in situ* hybridisation to examine DDR expression in an involved lymph node. Light and dark field images are on the left and right respectively. Specific probe (DDR) and control (Cont.) are shown. Oligonucleotide probes were used. Magnification x 200.

Figure 3 shows a Northern blot to examine DDR expression in human breast tissue. 10μg of total RNA per lane. Upper panel shows DDR transcript, lower panel ethidium bromide stained 28S

rRNA.

Figure 4 shows DDR expression in murine mammary development. 2μg Poly (A)+ RNA per lane. DDR transcript shown in upper panel. Blot was hybridised with GAPDH to demonstrate RNA loading (lower panel).

Figure 5 shows Norther blot analysis of DDR expression in human cell lines performed as for Figure 1. DDR transcript (upper panel), ethidium bromide stained 28S rRNA (lower panel).

Figure 6 shows *in situ* hybridisation analysis of DDR expression in human breast tumour tissue. Tissues were from three different individuals: a, b and c. In each panel, light and dark field images are on the left and right respectively. Specific probes are shown (DDR), and controls (Cont.). il= infiltrating lymphocytes, tu= tumour and ne= normal epithelium. Experiments 6a and 6b used oligonucleotides probes, and 6c used RNA probes. Magnification x 200.

Figure 7 shows DDR expression in normal tissues. a) human multiple tissue Norther blot containing 2μg poly (A)+ RNA per lane. DDR transcript shown in upper panel. Blot was stripped and rehybridised with human GAPDH probe to demonstrate RNA loading (lower panel). b) Total RNA Northern blot, 10μg RNA per lane, DDR transcript upper panel, ethidium bromide stained 28S rRNA lower panel.

MATERIALS AND METHODS

Tissues

Human tumour bearing or non-involved lymph nodes, tumour and normal tissue samples were obtained from surgery and rapidly frozen in liquid nitrogen. Normal breast tissue was obtained from reduction mammoplasty. Murine mammary tissue was obtained by excision of the fourth gland, removal of the associated lymph node, and rapid freezing in liquid nitrogen. All tissues were stored at -70°C or in liquid nitrogen.

Histopathological examination determined the presence or absence of metastases in the lymph nodes.

Cell lines

CAL 51, a human breast carcinoma cell line, was isolated and described by Gioanni *et al* (Br. J. Cancer, 62, 8-13 (1990)). The fibrosarcoma cell line HT1080, and the other breast cancer cell lines were purchased from the American Type Culture Collection. Cells were cultured according to conditions recommended by the suppliers.

RNA extraction

Total RNA was extracted from all tissues and cell lines by the method of Chomczynski and Sacchi (Anal. Biochem., 162, 156-159 (1987)). Poly (A)+ RNA was isolated from murine mammary gland total RNA samples using the Poly A+ Tract Kit (Promega) according to the manufacturer's instructions, except that mRNA was eluted with three washes (2 x 200μl, 1 x 100μl) of water.

RT-PCR and PTK differential screen

5μg of total RNA purified from involved or non-involved lymph nodes was digested with 10 units of RNase free DNase 1 (Pharmacia) using the manufacturer's recommended conditions in the presence of 40 units of RNase inhibitor (Boehringer Mannheim). Samples were heated to 100°C for 1 minute (min) to inactivate the DNase, extracted with phenol/chloroform and precipitated with ethanol. For cDNA synthesis, 2.5μg of RNA was resuspended in water and denatured at 65°C for 3 min in the presence of 40 ng $pd(N)_6$ random hexamers (Pharmacia) and cooled on ice. Reverse transcription was carried out using 1 mM dNTPs (Pharmacia), 40 units RNase inhibitor, 30 units of AMV reverse transcriptase SL (Life Sciences) and the buffer provided by the manufacturer at 41°C for 1 hour. Control reactions were performed as above but omitting the reverse transcriptase enzyme (RT-control). To inactivate the inhibitory properties of reverse transcriptase on Taq polymerase, samples were heated to 100°C for 1 min.

Degenerate oligonucleotides were designed to amplify by PCT

an approximately 200 bp region encoding part of the catalytic domain of PTKs. The highly conserved amino acid sequence shown in

SEQ ID NO. 3

was used for designing the 5' oligonucleotide shown in

SEQ ID NO. 4

and the highly conserved amino acid sequence shown in

SEQ ID NO. 5

was used for designing the 3' oligonucleotide shown in

SEQ ID NO. 6.

Amplifications were carried out using cDNA derived from 0.5μg of original RNA in 60 mM KCl, 15mM Tris-HCl, 1.1mM $MgCl_2$, 200μM of each dNTP, 0.5ng of each primer and 2 units of Taq polymerase (Cetus). The reaction mixtures were cycled 30 times at 94°C for 1 min, 55°C for 2 min and 72°C for 1 min. For each sample, PCR was carried out on cDNA and the relevant RT-control. The amplified products were electrophoresed through 3% agarose gels, the approximately 200 bp band excised and purified using a Mermaid kit (Bio 101). PCR products from involved nodes were subcloned into the pCR II vector (Invitrogen) according to the manufacturer's instructions. Clones were picked, grown in liquid culture, pelleted, resuspended in denaturing solution (0.5M NaOH, 1.5M NaCl) and aliquots were gridded onto duplicate Hybond-N membranes (Amersham). Filters were then neutralised in 3M NaCl, 0.5M Tris-HCl (pH 7.4). Duplicate filers were hybridised with ^{32}P labelled randomly primed (Feinberg & Vogelstein, Anal. Biochem, 132, 6-13 (1983); and Feinberg & Vogelstein, Anal. Biochem, 137, 266-267 (1984)) PCR products from either the involved or non-involved node. Clones containing PTK fragments that hybridised more strongly with the involved node-derived probe compared with the non-involved node derived probe were chosen for sequence analysis.

DNA sequencing

Templates for sequencing were prepared by the rapid method of Yie *et al* (Nucleic Acids Res., 21, 361(1993)) and were sequenced by the dideoxy method of Sanger *et al*, (Proc. Natl. Acad. Sci. (USA), 74, 5463-5467 (1977)) using a Sequenase

Version 2.0 kit (United States Biochemicals).

3' RACE-PCR

To isolate cDNA sequences 3' to the region amplified by the PTK PCR, a modification of the RACE protocol described by Frohman *et al* (Proc. Natl. Acad. Sci. (USA), 85, 8998-9002 (1988)) was used. RNA from human breast carcinoma cell line MDA MG 468 was reverse transcribed using the tagged oligo d(T) primer shown in

SEQ ID NO. 7.

Two nested sense oligonucleotides were used in consecutive PCR reactions. The upstream sense primer was as shown in

SEQ ID NO. 8

and the downstream sense primer was as shown in

SEQ ID NO. 9.

The tag sequence used with oligo d(T) for the reverse transcription was used as the antisense primer in both amplifications. The reaction buffer was as described for the PTK PCR except $MgCl_2$ was used at 1.5mM, cycling parameters were 94°C for 1 min 15 seconds, 56°C for 2 min and 72°C for 2 min.

Northern blots

Human total RNA samples were electrophoresed through formaldehyde/agarose gels, transferred to Hybond-N membranes (Amersham) and fixed by U.V. illumination. The human multiple tissue Northern blot was purchased from Clontech. Murine Poly (A)+ RNA samples were fractionated in agarose gels containing glyoxal/DMSO, transferred to ZetaProbe membranes (Biorad) and fixed by baking at 80°C for 2 hours. ^{32}P labelled probes were prepared as described by Feinberg & Vogelstein (supra). Hybridisations were carried out according to the manufacturer's instructions, membranes were washed to a final stringency of 0.1 x SSC, 0.1% SDS at 65°C.

Probe generation for *in situ* hybridisation

Riboprobes: The DDR RACE PCR fragment was sub-cloned into pBluescript KS^+(Stratagene) and sense and antisense RNAs labelled with ^{35}S-UTP (Amersham) were synthesised. The

transcription reactions were carried out using a Stratagene Transcription kit according to the manufacturer's instruction. Probes were subject to limited alkaline hydrolysis at 65°C for 100 min (Cox *et al.*, Developmental Biology, 101, 485-502 (1984)) to achieve a final length of between 100-200 bases. Full length and hydrolysed probes were examined on formaldehyde/agarose gels.

Oligonucleotide probes: Antisense 30mer oligonucleotides as follows were synthesised to the 3' untranslated and catalytic domain encoding regions of DDR:-

SEQ ID NO. 10
SEQ ID NO. 11
SEQ ID NO. 12
SEQ ID NO. 13.

A 30mer random oligonucleotide N_{30} was also synthesised (randomer).

The oligonucleotides were 3'-labelled with ^{33}P-dATP (Amersham) using terminal transferase according to manufacturer's instructions (Boehringer Mannheim). Probes were purified by passing through Chroma-spin 10 columns (Clontech) to remove unincorporated label. Tail lengths were analysed on a DNA sequencing gel. The four specific oligonucleotides were then mixed equally to form a cocktail.

Labelling efficiency of all probes was assessed by scintillation counting.

***In situ* hybridisation**

Except those used in post-hybridisation steps, all solutions were diethylpyrocarbonate treated and all glassware was baked.

7μm cryostat sections were cut from snap frozen human tumour material which had been excised during surgery, and stored in either liquid nitrogen or at -80°C. Sections were dried onto APTES (3-aminopropyltriethoxysilane) treated slides (Rentrop *et al.*, Histochem. J., 18, 271-276 (1986)) over dry ice, and fixed at 4°C in 4% paraformaldehyde, phosphate buffered saline

(PBS) for 15 min, followed by two 15 min washes in PBS. The sections were dehydrated through ethanols from 30%-100%, air dried, and stored at -80°C until required.

a) With riboprobes

Sections were equilibrated to room temperature for 5 min, quickly rehydrated though ethanols from 100% to 30%, and washed twice for 5 min in PBS. Sections were acetylated for 10 min in 0.1M triethanalomine, 0.25% acetic anhydride while stirring. After washing in 2xSSC for 2 min, denaturation of tissue RNAs was carried out in 50% formamide, 2xSSC for 10 min at 60°C. Sections were then dehydrated and air dried.

Hybridisation was performed overnight in buffer (50% formamide, 0.3M NaCl, 10mM Tris- HCl pH8, 2mM EDTA pH8, 10% dextran sulphate, 10mM sodium phosphate pH8, 50μgml^{-1}ssDNA) with comparable counts per minute (cpm) of either antisense or sense riboprobes, in a humid chamber at 50°C.

After hybridisation, two 30 min washes in 2xSSC, 50% formamide at 60°C were followed by treatment with 20μgml^{-1} RNAse A in 0.5M NaCl, 10mM Tris HCl pH7.5, 5mM EDTA pH 7.5 for 30 min at 37°C. Sections were washed for 15 min in 2xSSC at 60°C, followed by two final washes at a stringency of 0.1xSSC for 30 min at 60°C. Sections were dehydrated and air dried before autoradiography.

b) With oligonucleotide probes.

Sections were equilibrated to room temperature, rehydrated, washed and acetylated as described for sections hybridised with riboprobes. After washing twice for 5 min with PBS they were dehydrated and air dried.

Sections were overlaid with 10μl of hybridisation buffer (6x SSC, 50% formamide, 5x Denhardt's solution (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, USA (1989)), 10% dextran sulphate)

containing 20μgml^{-1} unlabelled random oligonucleotide, and prehybridised for 3 hours at 37°C in a sealed humid chamber. After prehybridisation, a further 10μl of hybridisation buffer containing comparable cpm of either the labelled oligonucleotide cocktail or labelled randomer were added to each section and gently mixed with prehybridisation solution. The sections were incubated overnight at 37°C (Lathe, *In Situ* Hybridisation Principles and Practice, Polak & McGee (eds.) Oxford Scientific Publications, Oxford pp71-80 (1989)) in a sealed humid chamber.

Sections were removed from the chamber, and the hybridisation solution was rinsed off with prewarmed 5xSSC gently pipetted over the slide. The area around the sections was dried and the sections were then overlaid with 20μl prehybridisation solution (20μgml^{-1} randomer) and incubated for 60 min as before. This process might help reduce background by replacing non-specifically bound oligonucleotide with random oligonucleotide. This step was repeated once. Slides were washed to a final stringency of 60% formamide, 5x SSC at 37°C. Sections were dehydrated in 70% and 100% ethanol and air dried.

Autoradiography on tissues

This process was the same for both riboprobe and oligonucleotide methods. In order to give a guideline of the length of time of exposure to photographic emulsion required, slides were placed against autoradiography film overnight. Slides were dipped in K.5 (Ilford) photographic emulsion diluted 1:1 with 2% glycerol, following manufacturer's guidelines. Slides were dried in an air-tight container with desiccant at room temperature for 2 hours and stored dry at 4°C, in a light-tight box for the required length of time. Slides were developed in D19 (Kodak), stopped in 1% acetic acid, 1% glycerol and fixed in 30% sodium thiosulphate. Sections were counter stained in 0.02% toluidine blue, mounted in DPX (Fisons) and examined by light and darkfield microscopy.

RESULTS

Identification of PTKs in a differential screen of involved and non-involved lymph nodes

RNA was isolated from involved and non-involved axillary lymph nodes from patients with metastatic breast tumours. RT-PCR was performed on the RNA using degenerate oligonucleotides encoding the amino acid sequences shown in SEQ ID NO. 3 and SEQ ID NO 5, sequences highly conserved in PTKs (Hanks, Science, 241, 42-52 (1988)). The resultant pools of approximately 200bp PTK cDNA fragments from the involved nodes were subcloned, and clones screened with mixed pools of radiolabelled PTK fragments from both involved and non-involved nodes. Clones amplified preferentially from the involved nodes were sequenced. For each of five involved lymph nodes, 50 or 100 clones were screened; differentially amplified cDNAs were found in four of five nodes. A novel kinase was detected as the major differentially amplified PTK-encoding cDNA; it showed similarities with trk A (Martin-Zanca et al, Nature, 319, 743-748 (1986)) and the rat PCR generated fragment tyro 10 (Lai & Lemke, Neuron, 6, 691-704 (1991)). The receptor PTK c-erb-B2 was found at high levels in three nodes.

The trk A/tyro 10-like PTK is identical to DDR

To study the expression patterns of the trk-like kinase, a 3' Rapid Amplification of cDNA Ends (RACE)-PCR fragment was generated using two specific nested oligonucleotides corresponding to the sequence between the two degenerate primers. Dideoxy sequencing of this 1.2 kilo base (kb) 3' RACE-PCR fragment revealed 384 nucleotides of predicted coding sequence. This comprised sequences encoding part of a putative PTK catalytic domain, which showed similarity to trk A, followed by a short C terminal domain of 8 amino acids after subdomain XI. While analysing this fragment, a report was published describing a receptor PTK found in cultured breast carcinoma cells lines with an extracellular discoidin 1-like domain (DDR) (Johnson et al, Proc. Natl. Acad. Sci. (USA), 90, 5677-5681 (1993)). Comparison of the DDR sequence

with the 3' RACE-PCR fragment revealed them to be identical, the 3' RACE-PCR fragment begins at nucleotide position 2500 of the published DDR sequence.

Expression of DDR mRNA in lymph nodes

DDR was detected in a PCR based differential screen designed to identity PTKs expressed at higher levels in tumour containing compared with tumour free lymph nodes. In order to examine whether higher levels of DDR mRNA were present in the involved compared with the non-involved lymph node, the 3' RACE-PCR fragment radiolabelled with ^{32}P was used as a probe to hybridise with Northern blots of lymph node RNA. This probe recognised a single band of approximately 4kb on Northern blots. DDR mRNA was undetectable in the non-involved lymph node and present in four of six metastasis-containing nodes (Figure 1). *In situ* hybridisation on a section of involved lymph node showed specific hybridisation over the metastasised tumour cells (Figure 2).

Expression of DDR mRNA in breast tumours, breast carcinoma cell lines and mammary development

The expression patterns of DDR in a panel of breast tumour RNAs and a number of breast carcinoma cell lines were examined by hybridising Northern blots with the ^{32}P labelled 3' RACE-PCR fragment. DDR mRNA was present in normal breast tissue and in all the breast tumours tested (figure 3). Elevated levels of expression were detected in samples of 'normal' breast and carcinoma, both surgically removed from a pregnant woman. To examine DDR expression in mammary gland development, a Northern blot of murine mammary gland poly (A)+ RNA was hybridised with the 3' RACE-PCR derived probe. Figure 4 shows that murine DDR mRNA (NEP, Zerlin et al, Oncogene, 8, 2731-2739 (1983)), see discussion) was detected in all stages of development. Expression was moderate in virgin glands, a slight increase was seen in mid-pregnancy, during lactation very low levels of DDR mRNA were detected, and moderate levels reappeared during involution. During lactation casein mRNA comprises a large proportion of the total, this effectively dilutes the levels of other mRNAs; glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) mRNA levels were decreased in these samples also. DDR mRNA expression was also detected in fat pad samples. The fat pad represents mammary tissue which has developed in the absence of epithelial cells due to the cauterisation of the nipple and surrounding tissue prior to epithelial outgrowth. Varying amounts of DDR mRNA were detected in the breast cancer cell lines examined, the highest were in MCF 7 and T47D and the lowest were in MDA MB 157 and BT 474. Expression was not detected in the fibrosarcoma cell line HT1080 (Figure 5).

Northern blot analysis revealed that DDR mRNA was present in normal breast and breast tumour tissue. *In situ* hybridisation was, therefore, performed on normal breast tissue and primary breast carcinoma samples to determine which cell types express DDR. In sections from three tumour samples, (Figures 6 a, b and c) hybridisation was predominantly over the tumour cells. DDR mRNA was undetectable in normal breast tissue adjacent to the tumour (Figure 6c top panel), and in all normal breast samples examined (data not shown). Figure 6c (top panel), shows a mass of invasive carcinoma surrounded by stromal cells in the top right of the field, with normal epithelial tissue in the bottom left of the field; hybridisation was specifically over the tumour cells, and DDR mRNA was undetectable in the normal epithelium and stromal cells. Figure 6c (middle panel) shows DDR positive cancer cells which have replaced the normal epithelial cells in the breast lobules. The malignant epithelial elements are surrounded by a dense lymphocytic infiltrate in which DDR was undetectable. A comparable result to Figure 6c was obtained using oligonucleotide probes, (date not shown). Figures 6a and c (lower panels) show results obtained with the random oligonucleotide probe and the sense riboprobe respectively, with no specific hybridisation. These controls were carried out for all *in situ* hybridisations (not shown in all cases).

Patterns of DDR expression in normal human tissues

A human poly (A)+ RNA multiple tissue Northern blot was screened with the 3′ RACE-PCR probe (Figure 7a). DDR was

expressed at high levels in kidney and placental tissue, low levels were detected in the heart and lung, and expression was undetectable in the liver. A more extensive panel of human tissues was examined on total RNA Northern blots (Figure 7b). DDR was expressed in a wide range of tissues, with relatively high levels in the kidney cortex and thyroid. By this analysis, the haematopoeitic tissues bone marrow and spleen did not have detectable levels of DDR mRNA.

DISCUSSION

The initial differential screen identified DDR as a PTK expressed in involved but not in non-involved lymph nodes from breast cancer patients. Further studies revealed varying levels of DDR mRNA in both normal breast and all of the breast carcinomas analysed. Elevated mRNA levels, relative to normal breast tissue, were found in both apparently normal breast tissue and breast carcinoma taken from the same pregnant patient. However, an examination of murine mammary gland development revealed only a moderate increase in DDR mRNA in pregnancy. The higher levels of DDR in the human pregnant breast could be due to a focus of infiltrating carcinoma, as the specimen was taken from the tissue adjacent to a tumour. Alternatively, the murine mammary gland may differ from human with respect to DDR expression during pregnancy. Unfortunately material was not available from the human pregnant breast for histophthalogical examination or *in situ* hybridisation studies to resolve this issue. *In situ* hybridisation studies were performed to determine which cells in a particular tissue sample express DDR mRNA, and to give an indication of the relative abundance of mRNA expressed by different cell types. *In situ* hybridisation of a heavily infiltrated lymph node revealed DDR mRNA at high levels in the metastasising tumour cells and undetectable levels in the adjacent cells. DDR mRNA was also shown to be present in tumour cells in primary breast carcinomas by *in situ* hybridisation. Where a direct comparison could be made, high levels of DDR mRNA were detected in the tumour cells but not in adjacent normal epithelial cells nor in tumour infiltrating

lymphocytes. By Northern analysis, DDR mRNA was present in normal human breast tissue, but by *in situ* hybridisation on human mammary tissue DDR has only been detected in tumour cells, which are epithelial in origin, and not in normal human breast epithelial or stromal cells. The question of the site of DDR expression in normal human mammary tissue is therefore unanswered; most likely cellular mRNA levels are too low for detection by *in situ* hybridisation. Nonetheless over expression of DDR occurs in some breast tumour cells when compared with normal mammary epithelium. Having demonstrated the presence of DDR mRNA in several sources of breast tumour tissue (in primary tumours, metastatic cells and carcinoma-derived cell lines) the expression in normal tissues was examined by Northern blotting. A wide range of levels were observed, ranging from high in the kidney cortex and thyroid, moderate in breast and lung, to undetectable amounts in the bond marrow, spleen, and liver. DDR expression is not restricted to any one tissue type, therefore, its role is not tissue specific. However, it is not ubiquitously expressed, thus a housekeeping function is unlikely and a cell specific role is probable.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The Institute of Cancer Research
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(C) CITY: London
(E) COUNTRY: Great Britain
(F) POSTAL CODE (ZIP): NW1 2BP

(ii) TITLE OF INVENTION: Cell Growth Factor Receptors

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3754 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 142..2883

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CCCGGGTCGG ACCGCCTGGG TCTGCCGGGA AGAGCGATGA GAGGTGTCTG AAGGTGGCTA      60

TTCACTGAGC GATGGGGTTG GACTTGAAGG AATGCCAAGA GATGCTGCCC CCACCCCCTT     120

AGGCCCGAGG GATCAGGAGC T ATG GGA CCA GAG GCC CTG TCA TCT TTA CTG        171
                       Met Gly Pro Glu Ala Leu Ser Ser Leu Leu
                         1               5                  10

CTG CTG CTC TTG GTG GCA AGT GGA GAT GCT GAC ATG AAG GGA CAT TTT        219
Leu Leu Leu Leu Val Ala Ser Gly Asp Ala Asp Met Lys Gly His Phe
                15                  20                  25

GAT CCT GCC AAG TGC CGC TAT GCC CTG GGC ATG CAG GAC CGG ACC ATC        267
Asp Pro Ala Lys Cys Arg Tyr Ala Leu Gly Met Gln Asp Arg Thr Ile
            30                  35                  40

CCA GAC AGT GAC ATC TCT GCT TCC AGC TCC TGG TCA GAT TCC ACT GCC        315
Pro Asp Ser Asp Ile Ser Ala Ser Ser Ser Trp Ser Asp Ser Thr Ala
        45                  50                  55
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GCC CGC CAC AGC AGG TTG GAG AGC AGT GAC GGG GAT GGG GCC TGG TGC      363
Ala Arg His Ser Arg Leu Glu Ser Ser Asp Gly Asp Gly Ala Trp Cys
 60                  65                  70

CCC GCA GGG TCG GTG TTT CCC AAG GAG GAG GAG TAC TTG CAG GTG GAT      411
Pro Ala Gly Ser Val Phe Pro Lys Glu Glu Glu Tyr Leu Gln Val Asp
 75                  80                  85                  90

CTA CAA CGA CTC CAC CTG GTG GCT CTG GTG GGC ACC CAG GGA CGG CAT      459
Leu Gln Arg Leu His Leu Val Ala Leu Val Gly Thr Gln Gly Arg His
                 95                 100                 105

GCC GGG GGC CTG GGC AAG GAG TTC TCC CGG AGC TAC CGG CTG CGT TAC      507
Ala Gly Gly Leu Gly Lys Glu Phe Ser Arg Ser Tyr Arg Leu Arg Tyr
            110                 115                 120

TCC CGG GAT GGT CGC CGC TGG ATG GGC TGG AAG GAC CGC TGG GGT CAG      555
Ser Arg Asp Gly Arg Arg Trp Met Gly Trp Lys Asp Arg Trp Gly Gln
        125                 130                 135

GAG GTG ATC TCA GGC AAT GAG GAC CCT GAG GGA GTG GTG CTG AAG GAC      603
Glu Val Ile Ser Gly Asn Glu Asp Pro Glu Gly Val Val Leu Lys Asp
    140                 145                 150

CTT GGG CCC CCC ATG GTT GCC CGA CTG GTT CGC TTC TAC CCC CGG GCT      651
Leu Gly Pro Pro Met Val Ala Arg Leu Val Arg Phe Tyr Pro Arg Ala
155                 160                 165                 170

GAC CGG GTC ATG AGC GTC TGT CTG CGG GTA GAG CTC TAT GGC TGC CTC      699
Asp Arg Val Met Ser Val Cys Leu Arg Val Glu Leu Tyr Gly Cys Leu
                175                 180                 185

TGG AGG GAT GGA CTC CTG TCT TAC ACC GCC CCT GTG GGG CAG ACA ATG      747
Trp Arg Asp Gly Leu Leu Ser Tyr Thr Ala Pro Val Gly Gln Thr Met
            190                 195                 200

TAT TTA TCT GAG GCC GTG TAC CTC AAC GAC TCC ACC TAT GAC GGA CAT      795
Tyr Leu Ser Glu Ala Val Tyr Leu Asn Asp Ser Thr Tyr Asp Gly His
        205                 210                 215

ACC GTG GGC GGA CTG CAG TAT GGG GGT CTG GGC CAG CTG GCA GAT GGT      843
Thr Val Gly Gly Leu Gln Tyr Gly Gly Leu Gly Gln Leu Ala Asp Gly
    220                 225                 230

GTG GTG GGG CTG GAT GAC TTT AGG AAG AGT CAG GAG CTG CGG GTC TGG      891
Val Val Gly Leu Asp Asp Phe Arg Lys Ser Gln Glu Leu Arg Val Trp
235                 240                 245                 250

CCA GGC TAT GAC TAT GTG GGA TGG AGC AAC CAC AGC TTC TCC AGT GGC      939
Pro Gly Tyr Asp Tyr Val Gly Trp Ser Asn His Ser Phe Ser Ser Gly
                255                 260                 265

TAT GTG GAG ATG GAG TTT GAG TTT GAC CGG CTG AGG GCC TTC CAG GCT      987
Tyr Val Glu Met Glu Phe Glu Phe Asp Arg Leu Arg Ala Phe Gln Ala
            270                 275                 280

ATG CAG GTC CAC TGT AAC AAC ATG CAC ACG CTG GGA GCC CGT CTG CCT     1035
Met Gln Val His Cys Asn Asn Met His Thr Leu Gly Ala Arg Leu Pro
        285                 290                 295

GGC GGG GTG GAA TGT CGC TTC CGG CGT GGC CCT GCC ATG GCC TGG GAG     1083
Gly Gly Val Glu Cys Arg Phe Arg Arg Gly Pro Ala Met Ala Trp Glu
    300                 305                 310

GGG GAG CCC ATG CGC CAC AAC CTA GGG GGC AAC CTG GGG GAC CCC AGA     1131
Gly Glu Pro Met Arg His Asn Leu Gly Gly Asn Leu Gly Asp Pro Arg
315                 320                 325                 330
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GCC CGG GCT GTC TCA GTG CCC CTT GGC GGC CGT GTG GCT CGC TTT CTG      1179
Ala Arg Ala Val Ser Val Pro Leu Gly Gly Arg Val Ala Arg Phe Leu
                335                 340                 345

CAG TGC CGC TTC CTC TTT GCG GGG CCC TGG TTA CTC TTC AGC GAA ATC      1227
Gln Cys Arg Phe Leu Phe Ala Gly Pro Trp Leu Leu Phe Ser Glu Ile
            350                 355                 360

TCC TTC ATC TCT GAT GTG GTG AAC AAT TCC TCT CCG GCA CTG GGA GGC      1275
Ser Phe Ile Ser Asp Val Val Asn Asn Ser Ser Pro Ala Leu Gly Gly
        365                 370                 375

ACC TTC CCG CCA GCC CCC TGG TGG CCG CCT GGC CCA CCT CCC ACC AAC      1323
Thr Phe Pro Pro Ala Pro Trp Trp Pro Pro Gly Pro Pro Pro Thr Asn
    380                 385                 390

TTC AGC AGC TTG GAG CTG GAG CCC AGA GGC CAG CCA AGG CCC GTG GCC      1371
Phe Ser Ser Leu Glu Leu Glu Pro Arg Gly Gln Pro Arg Pro Val Ala
395                 400                 405                 410

AAG GCC GAG GGG AGC CCG ACC GCC ATC CTC ATC GGC TGC CTG GTG GCC      1419
Lys Ala Glu Gly Ser Pro Thr Ala Ile Leu Ile Gly Cys Leu Val Ala
                415                 420                 425

ATC ATC CTG CTC CTG CTG CTC ATC ATT GCC CTC ATG CTC TGG CGG CTG      1467
Ile Ile Leu Leu Leu Leu Leu Ile Ile Ala Leu Met Leu Trp Arg Leu
            430                 435                 440

CAC TGG CGC AGG CTC CTC AGC AAG GCT GAA CGG AGG GTG TTG GAA GAG      1515
His Trp Arg Arg Leu Leu Ser Lys Ala Glu Arg Arg Val Leu Glu Glu
        445                 450                 455

GAG CTG ACG GTT CAC CTC TCT GTC CCT GGG GAC ACT ATC CTC ATC AAC      1563
Glu Leu Thr Val His Leu Ser Val Pro Gly Asp Thr Ile Leu Ile Asn
    460                 465                 470

AAC CGC CCA GGT CCT AGA GAG CCA CCC CCG TAC CAG GAG CCC CGG CCT      1611
Asn Arg Pro Gly Pro Arg Glu Pro Pro Pro Tyr Gln Glu Pro Arg Pro
475                 480                 485                 490

CGT GGG AAT CCG CCC CAC TCC GCT CCC TGT GTC CCC AAT GGC TCT GCG      1659
Arg Gly Asn Pro Pro His Ser Ala Pro Cys Val Pro Asn Gly Ser Ala
                495                 500                 505

TTG CTG CTC TCC AAT CCA GCC TAC CGC CTC CTT CTG GCC ACT TAC GCC      1707
Leu Leu Leu Ser Asn Pro Ala Tyr Arg Leu Leu Leu Ala Thr Tyr Ala
            510                 515                 520

CGT CCC CCT CGA GGC CCG GGC CCC CCC ACA CCC GCC TGG GCC AAA CCC      1755
Arg Pro Pro Arg Gly Pro Gly Pro Pro Thr Pro Ala Trp Ala Lys Pro
        525                 530                 535

ACC AAC ACC CAG GCC TAC AGT GGG GAC TAT ATG GAG CCT GAG AAG CCA      1803
Thr Asn Thr Gln Ala Tyr Ser Gly Asp Tyr Met Glu Pro Glu Lys Pro
    540                 545                 550

GGC GCC CCG CTT CTG CCC CCA CCT CCC CAG AAC AGC GTC CCC CAT TAT      1851
Gly Ala Pro Leu Leu Pro Pro Pro Pro Gln Asn Ser Val Pro His Tyr
555                 560                 565                 570

GCC GAG GCT GAC ATT GTT ACC CTG CAG GGC GTC ACC GGG GGC AAC ACC      1899
Ala Glu Ala Asp Ile Val Thr Leu Gln Gly Val Thr Gly Gly Asn Thr
                575                 580                 585

TAT GCT GTG CCT GCA CTG CCC CCA GGG GCA GTC GGG GAT GGG CCC CCC      1947
Tyr Ala Val Pro Ala Leu Pro Pro Gly Ala Val Gly Asp Gly Pro Pro
            590                 595                 600
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AGA GTG GAT TTC CCT CGA TCT CGA CTC CGC TTC AAG GAG AAG CTT GGC      1995
Arg Val Asp Phe Pro Arg Ser Arg Leu Arg Phe Lys Glu Lys Leu Gly
      605                 610                 615

GAG GGC CAG TTT GGG GAG GTG CAC CTG TGT GAG GTC GAC AGC CCT CAA      2043
Glu Gly Gln Phe Gly Glu Val His Leu Cys Glu Val Asp Ser Pro Gln
  620                 625                 630

GAT CTG GTT AGT CTT GAT TTC CCC CTT AAT GTG CGT AAG GGA CAC CCT      2091
Asp Leu Val Ser Leu Asp Phe Pro Leu Asn Val Arg Lys Gly His Pro
635                 640                 645                 650

TTG CTG GTA GCT GTC AAG ATC TTA CGG CCA GAT GCC ACC AAG AAT GCC      2139
Leu Leu Val Ala Val Lys Ile Leu Arg Pro Asp Ala Thr Lys Asn Ala
                655                 660                 665

AGG AAT GAT TTC CTG AAA GAG GTG AAG ATC ATG TCG AGG CTC AAG GAC      2187
Arg Asn Asp Phe Leu Lys Glu Val Lys Ile Met Ser Arg Leu Lys Asp
          670                 675                 680

CCA AAC ATC ATT CGG CTG CTG GGC GTG TGT GTG CAG GAC GAC CCC CTC      2235
Pro Asn Ile Ile Arg Leu Leu Gly Val Cys Val Gln Asp Asp Pro Leu
      685                 690                 695

TGC ATG ATT ACT GAC TAC ATG GAG AAC GGC GAC CTC AAC CAG TTC CTC      2283
Cys Met Ile Thr Asp Tyr Met Glu Asn Gly Asp Leu Asn Gln Phe Leu
  700                 705                 710

AGT GCC CAC CAG CTG GAG GAC AAG GCA GCC GAG GGG GCC CCT GGG GAC      2331
Ser Ala His Gln Leu Glu Asp Lys Ala Ala Glu Gly Ala Pro Gly Asp
715                 720                 725                 730

GGG CAG GCT GCG CAG GGG CCC ACC ATC AGC TAC CCA ATG CTG CTG CAT      2379
Gly Gln Ala Ala Gln Gly Pro Thr Ile Ser Tyr Pro Met Leu Leu His
                735                 740                 745

GTG GCA GCC CAG ATC GCC TCC GGC ATG CGC TAT CTG GCC ACA CTC AAC      2427
Val Ala Ala Gln Ile Ala Ser Gly Met Arg Tyr Leu Ala Thr Leu Asn
          750                 755                 760

TTT GTA CAT CGG GAC CTG GCC ACG CGG AAC TGC CTA GTT GGG GAA AAT      2475
Phe Val His Arg Asp Leu Ala Thr Arg Asn Cys Leu Val Gly Glu Asn
      765                 770                 775

TTC ACC ATC AAA ATC GCA GAC TTT GGC ATG AGC CGG AAC CTC TAT GCT      2523
Phe Thr Ile Lys Ile Ala Asp Phe Gly Met Ser Arg Asn Leu Tyr Ala
  780                 785                 790

GGG GAC TAT TAC CGT GTG CAG GGC CGG GCA GTG CTG CCC ATC CGC TGG      2571
Gly Asp Tyr Tyr Arg Val Gln Gly Arg Ala Val Leu Pro Ile Arg Trp
795                 800                 805                 810

ATG GCC TGG GAG TGC ATC CTC ATG GGG AAG TTC ACG ACT GCG AGT GAC      2619
Met Ala Trp Glu Cys Ile Leu Met Gly Lys Phe Thr Thr Ala Ser Asp
                815                 820                 825

GTG TGG GCC TTT GGT GTG ACC CTG TGG GAG GTG CTG ATG CTC TGT AGG      2667
Val Trp Ala Phe Gly Val Thr Leu Trp Glu Val Leu Met Leu Cys Arg
          830                 835                 840

GCC CAG CCC TTT GGG CAG CTC ACC GAC GAG CAG GTC ATC GAG AAC GCG      2715
Ala Gln Pro Phe Gly Gln Leu Thr Asp Glu Gln Val Ile Glu Asn Ala
      845                 850                 855

GGG GAG TTC TTC CGG GAC CAG GGC CGG CAG GTG TAC CTG TCC CGG CCG      2763
Gly Glu Phe Phe Arg Asp Gln Gly Arg Gln Val Tyr Leu Ser Arg Pro
  860                 865                 870
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CCT GCC TGC CCG CAG GGC CTA TAT GAG CTG ATG CTT CGG TGC TGG AGC      2811
Pro Ala Cys Pro Gln Gly Leu Tyr Glu Leu Met Leu Arg Cys Trp Ser
875                 880                 885                 890

CGG GAG TCT GAG CAG CGA CCA CCC TTT TCC CAG CTG CAT CGG TTC CTG      2859
Arg Glu Ser Glu Gln Arg Pro Pro Phe Ser Gln Leu His Arg Phe Leu
                895                 900                 905

GCA GAG GAT GCA CTC AAC ACG GTG TGAATCACAC ATCCAGCTGC CCCTCCCTCA      2913
Ala Glu Asp Ala Leu Asn Thr Val
            910

GGGAGCGATC CAGGGGAAGC CAGTGACACT AAAACAAGAG GACACAATGG CACCTCTGCC     2973

CCTTCCCCTC CCGACAGCCC ATCACCTCTA ATAGAGGCAG TGAGACTGCA GGCTGGGCCC     3033

ACCCAGGGAG CTGATGCCCC TTCTCCCCTT CCTGGACACA CTCTCATGTC CCCTTCCTGT     3093

TCTTCCTTCC TAGAAGCCCC TGTCGCCCAC CCAGCTGGTC CTGTGGATGG GATCCTCTCC     3153

ACCCACCTCT AGCCATCCCT TGGGGAAGGG TGGGGAGAAA TATAGGATAG ACACTGGACA     3213

TGGCCCATTG GAGCACCTGG GCCCCACTGG ACAACACTGA TTCCTGGACA GGTGGCTGCG     3273

CCCCCAGCTT CTCTCTCCCT GTCACACACT GGACCCCACT GGCTGAGAAT CTGGGGGTGA     3333

GGAGGACAAG AAGGAGAGGA AAATGTTTCC TTGTGCCTGC TCCTGTACTT GTCCTCAGCT     3393

TGGGCTTCTT CCTCCTCCAT CACCTGAAAC ACTGGACCTG GGGGTAGCCC CGCCCCAGCC     3453

CTCAGTCACC CCCCACTTCC CACCTGCAGT CTTGTAGCTA GAACTTCTCT AAGCCTATAC     3513

GTTTCTGTGG AGTAAATATT GGGATTGGGG GGAAAGAGGG AGCAACGGCC CATAGCCTTG     3573

GGGTTGGACA TCTCTAGTGT AGCTGCCACA TTGATTTTTC TATAATCACT TGGGTTTGTA     3633

CATTTTTGGG GGGAGAGACA CAGATTTTTA CACTAATATA TGGACCTAGC TTGAGGCAAT     3693

TTTAATCCCC TGCACTAGGC AGGTAATAAT AAAGGTTGAG TTTTCCACAA AAAAAAAAAA     3753

A                                                                    3754
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 914 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Gly Pro Glu Ala Leu Ser Ser Leu Leu Leu Leu Leu Val Ala
 1               5                  10                  15

Ser Gly Asp Ala Asp Met Lys Gly His Phe Asp Pro Ala Lys Cys Arg
            20                  25                  30

Tyr Ala Leu Gly Met Gln Asp Arg Thr Ile Pro Asp Ser Asp Ile Ser
        35                  40                  45

Ala Ser Ser Ser Trp Ser Asp Ser Thr Ala Ala Arg His Ser Arg Leu
    50                  55                  60

Glu Ser Ser Asp Gly Asp Gly Ala Trp Cys Pro Ala Gly Ser Val Phe
65                  70                  75                  80
```

```
Pro Lys Glu Glu Glu Tyr Leu Gln Val Asp Leu Gln Arg Leu His Leu
                85                  90                  95

Val Ala Leu Val Gly Thr Gln Gly Arg His Ala Gly Gly Leu Gly Lys
            100                 105                 110

Glu Phe Ser Arg Ser Tyr Arg Leu Arg Tyr Ser Arg Asp Gly Arg Arg
        115                 120                 125

Trp Met Gly Trp Lys Asp Arg Trp Gly Gln Glu Val Ile Ser Gly Asn
    130                 135                 140

Glu Asp Pro Glu Gly Val Val Leu Lys Asp Leu Gly Pro Pro Met Val
145                 150                 155                 160

Ala Arg Leu Val Arg Phe Tyr Pro Arg Ala Asp Arg Val Met Ser Val
                165                 170                 175

Cys Leu Arg Val Glu Leu Tyr Gly Cys Leu Trp Arg Asp Gly Leu Leu
            180                 185                 190

Ser Tyr Thr Ala Pro Val Gly Gln Thr Met Tyr Leu Ser Glu Ala Val
        195                 200                 205

Tyr Leu Asn Asp Ser Thr Tyr Asp Gly His Thr Val Gly Gly Leu Gln
    210                 215                 220

Tyr Gly Gly Leu Gly Gln Leu Ala Asp Gly Val Val Gly Leu Asp Asp
225                 230                 235                 240

Phe Arg Lys Ser Gln Glu Leu Arg Val Trp Pro Gly Tyr Asp Tyr Val
                245                 250                 255

Gly Trp Ser Asn His Ser Phe Ser Ser Gly Tyr Val Glu Met Glu Phe
            260                 265                 270

Glu Phe Asp Arg Leu Arg Ala Phe Gln Ala Met Gln Val His Cys Asn
        275                 280                 285

Asn Met His Thr Leu Gly Ala Arg Leu Pro Gly Gly Val Glu Cys Arg
    290                 295                 300

Phe Arg Arg Gly Pro Ala Met Ala Trp Glu Gly Glu Pro Met Arg His
305                 310                 315                 320

Asn Leu Gly Gly Asn Leu Gly Asp Pro Arg Ala Arg Ala Val Ser Val
                325                 330                 335

Pro Leu Gly Gly Arg Val Ala Arg Phe Leu Gln Cys Arg Phe Leu Phe
            340                 345                 350

Ala Gly Pro Trp Leu Leu Phe Ser Glu Ile Ser Phe Ile Ser Asp Val
        355                 360                 365

Val Asn Asn Ser Ser Pro Ala Leu Gly Gly Thr Phe Pro Pro Ala Pro
    370                 375                 380

Trp Trp Pro Pro Gly Pro Pro Pro Thr Asn Phe Ser Ser Leu Glu Leu
385                 390                 395                 400

Glu Pro Arg Gly Gln Pro Arg Pro Val Ala Lys Ala Glu Gly Ser Pro
                405                 410                 415

Thr Ala Ile Leu Ile Gly Cys Leu Val Ala Ile Ile Leu Leu Leu Leu
            420                 425                 430

Leu Ile Ile Ala Leu Met Leu Trp Arg Leu His Trp Arg Arg Leu Leu
        435                 440                 445
```

```
Ser Lys Ala Glu Arg Arg Val Leu Glu Glu Glu Leu Thr Val His Leu
    450                 455                 460

Ser Val Pro Gly Asp Thr Ile Leu Ile Asn Asn Arg Pro Gly Pro Arg
465                 470                 475                 480

Glu Pro Pro Pro Tyr Gln Glu Pro Arg Pro Arg Gly Asn Pro Pro His
                485                 490                 495

Ser Ala Pro Cys Val Pro Asn Gly Ser Ala Leu Leu Leu Ser Asn Pro
            500                 505                 510

Ala Tyr Arg Leu Leu Leu Ala Thr Tyr Ala Arg Pro Pro Arg Gly Pro
        515                 520                 525

Gly Pro Pro Thr Pro Ala Trp Ala Lys Pro Thr Asn Thr Gln Ala Tyr
    530                 535                 540

Ser Gly Asp Tyr Met Glu Pro Glu Lys Pro Gly Ala Pro Leu Leu Pro
545                 550                 555                 560

Pro Pro Pro Gln Asn Ser Val Pro His Tyr Ala Glu Ala Asp Ile Val
                565                 570                 575

Thr Leu Gln Gly Val Thr Gly Gly Asn Thr Tyr Ala Val Pro Ala Leu
            580                 585                 590

Pro Pro Gly Ala Val Gly Asp Gly Pro Pro Arg Val Asp Phe Pro Arg
        595                 600                 605

Ser Arg Leu Arg Phe Lys Glu Lys Leu Gly Glu Gly Gln Phe Gly Glu
    610                 615                 620

Val His Leu Cys Glu Val Asp Ser Pro Gln Asp Leu Val Ser Leu Asp
625                 630                 635                 640

Phe Pro Leu Asn Val Arg Lys Gly His Pro Leu Leu Val Ala Val Lys
                645                 650                 655

Ile Leu Arg Pro Asp Ala Thr Lys Asn Ala Arg Asn Asp Phe Leu Lys
            660                 665                 670

Glu Val Lys Ile Met Ser Arg Leu Lys Asp Pro Asn Ile Ile Arg Leu
        675                 680                 685

Leu Gly Val Cys Val Gln Asp Asp Pro Leu Cys Met Ile Thr Asp Tyr
    690                 695                 700

Met Glu Asn Gly Asp Leu Asn Gln Phe Leu Ser Ala His Gln Leu Glu
705                 710                 715                 720

Asp Lys Ala Ala Glu Gly Ala Pro Gly Asp Gly Gln Ala Ala Gln Gly
                725                 730                 735

Pro Thr Ile Ser Tyr Pro Met Leu Leu His Val Ala Ala Gln Ile Ala
            740                 745                 750

Ser Gly Met Arg Tyr Leu Ala Thr Leu Asn Phe Val His Arg Asp Leu
        755                 760                 765

Ala Thr Arg Asn Cys Leu Val Gly Glu Asn Phe Thr Ile Lys Ile Ala
    770                 775                 780

Asp Phe Gly Met Ser Arg Asn Leu Tyr Ala Gly Asp Tyr Tyr Arg Val
785                 790                 795                 800

Gln Gly Arg Ala Val Leu Pro Ile Arg Trp Met Ala Trp Glu Cys Ile
                805                 810                 815
```

Leu Met Gly Lys Phe Thr Thr Ala Ser Asp Val Trp Ala Phe Gly Val
820 825 830

Thr Leu Trp Glu Val Leu Met Leu Cys Arg Ala Gln Pro Phe Gly Gln
835 840 845

Leu Thr Asp Glu Gln Val Ile Glu Asn Ala Gly Glu Phe Phe Arg Asp
850 855 860

Gln Gly Arg Gln Val Tyr Leu Ser Arg Pro Pro Ala Cys Pro Gln Gly
865 870 875 880

Leu Tyr Glu Leu Met Leu Arg Cys Trp Ser Arg Glu Ser Glu Gln Arg
885 890 895

Pro Pro Phe Ser Gln Leu His Arg Phe Leu Ala Glu Asp Ala Leu Asn
900 905 910

Thr Val

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Asp Leu Ala Ala Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGAATTCTAG AMGSGACYTG GCVGCBAGRA AC 32

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5..one-of(2)
(D) OTHER INFORMATION: /note= "Xaa at position 5 is Tyr or Phe"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7..one-of(2)
(D) OTHER INFORMATION: /note= "Xaa at position 7 is Val or Ile"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asp Val Trp Ser Xaa Gly Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGAATTCTAG ACACSCCRWA RSWCCASACR TC 32

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GACTCGAGTC GACATCGATT TTTTTTTTTT TTTTT 35

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTGGGGAAA ATTTCACCAT C 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGCATGAGCC GGAACCTC 18

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TCTCGATGAC CTGCTCGTCG GTGAGCTGCC 30

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CAGTCTCACT GCCTCTATTA GAGGTGATGG 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AAGCTGAGGA CAAGTACAGG AGCAGGCACA 30

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TCAATGTGGC AGCTACACTA GAGATGTCCA 30

CLAIMS:

1. A method of screening a substance for potential utility as a therapeutic agent in the treatment of cancer which comprises providing a standard system in which a protein tyrosine kinase or an active fragment thereof is able to develop a measurable effect, allowing the protein tyrosine kinase to develop that effect in the presence and absence of the said substance and measuring that effect, ability to produce significant inhibition of the effect being taken as an indication of potential utility as a therapeutic agent, wherein the protein tyrosine kinase is characterised by the amino acid sequence shown in

SEQ ID NO. 1

or an amino acid sequence showing a significant degree of homology thereto.

2. A method as claimed in Claim 1 wherein the protein tyrosine kinase or an active fragment thereof is derived from a recombinant expression system or a cellular source in which the protein is overexpressed.

3. A method as claimed in claim 2 wherein the recombinant expression system is an insect baculovirus, an immortalised human breast cell line or a rodent fibroblast cell line.

4. A method as claimed in claim 2 or 3 wherein the measurable effect is protein tyrosine kinase activity which is measured by measuring autophosphorylation or phosphorylation of a model substrate, by following incorporation of radioactive phosphate or by using anti-phosphotyrosine antibodies.

5. A method as claimed in Claim 1 wherein the measurable effect is a phenotypic alteration conferred by expression of the protein tyrosine kinase or an active fragment thereof in a recombinant or non-recombinant cell line.

6. A method as claimed in Claim 1 wherein the measurable effect is the tumorogenic effect of a recombinant or non-recombinant cell line expressing the protein tyrosine kinase or an active fragment thereof when used as a xenograft in an animal.

7. A method as claimed in Claim 5 or 6 wherein the recombinant cell line is a primary immortalised human breast cell line or a rodent fibroblast cell line.

8. A therapeutic agent identified by a method as claimed in any of Claims 1 to 7.

9. A therapeutic agent as claimed in Claim 8 which is a chemical molecule of relatively low molecular weight.

10. A therapeutic agent as claimed in Claim 9 which is a staurosporine analogue, a tyrphostin or a flavenoid.

11. A therapeutic agent as claimed in Claim 8 which is a macromolecule.

12. A therapeutic agent as claimed in Claim 11 which is an antibody.

13. A therapeutic agent as claimed in claim 8 which is a peptide.

14. A therapeutic agent as claimed in claim 8 which is an antisense oligonucleotide capable of blocking translation of the mRNA encoding the protein tyrosine kinase.

15. A method for detecting a tyrosine kinase having an amino acid sequence as defined in

SEQ ID NO. 1

or a fragment thereof which comprises contacting a test sample with a specific antibody raised against an antigen from the

said amino acid sequence and determining whether there is any antigen antibody binding within the test sample.

16. A method for the detection in a sample of a nucleic acid sequence encoding a tyrosine kinase which comprises subjecting the sample or nucleic acid isolated therefrom to a method capable of detecting a nucleic acid sequence therein identical or substantially homologous to a defined nucleic acid sequence and identifying any sequence identical or substantially homologous to all or part of the DNA sequence defined in

SEQ ID NO. 1,

or mRNA which would be the transcription product of such DNA.

17. A method as claimed in Claim 16 wherein DNA is detected by Southern blotting and/or PCR using appropriate primers.

18. A method as claimed in Claim 16 wherein RNA is detected by Northern blotting, RNAse protection studies and direct nucleic acid *in situ* hybridisation.

19. An enzyme-substrate complex which comprises a protein tyrosine kinase characterised by the amino acid sequence shown in

SEQ ID NO. 1

or an amino acid sequence showing a significant degree of homology thereto and a therapeutic agent capable of modulating the activity of said protein tyrosine kinase.

20. A complex as claimed in Claim 19 wherein the therapeutic agent is a chemical molecule of relatively low molecular weight.

21. A complex as claimed in Claim 20 wherein the therapeutic agent is a staurosporine analogue, a tyrphostin or a flavenoid.

22. A complex as claimed in Claim 19 wherein the therapeutic

agent is a macromolecule.

23. A complex as claimed in Claim 22 wherein the therapeutic agent is an antibody.

24. A complex as claimed in Claim 19 wherein the therapeutic agent as a peptide.

25. A complex as claimed in Claim 19 wherein the therapeutic agent is an antisense oligonucleotide capable of blocking translation of the mRNA encoding the protein tyrosine kinase.

FIG. 1

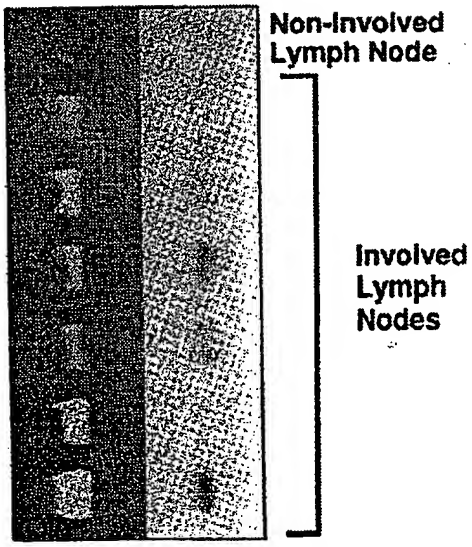


FIG. 2

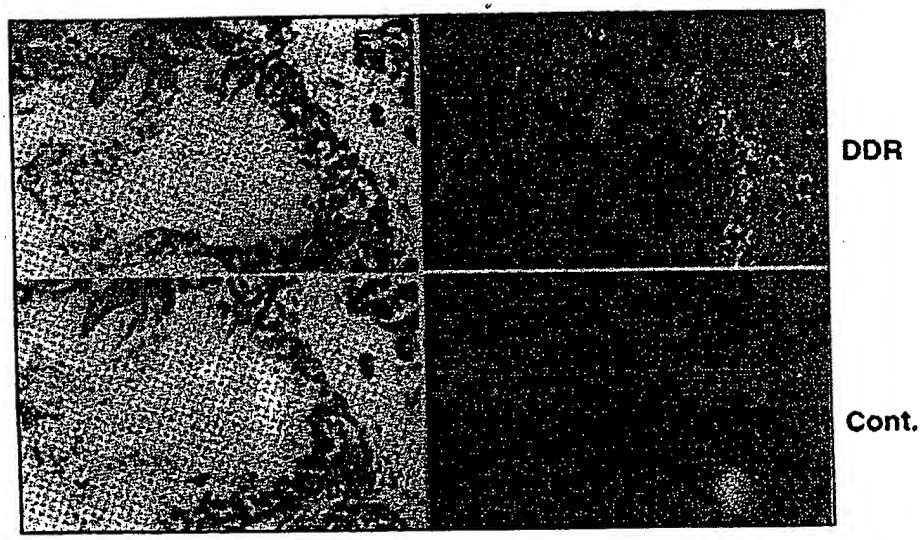


FIG. 4

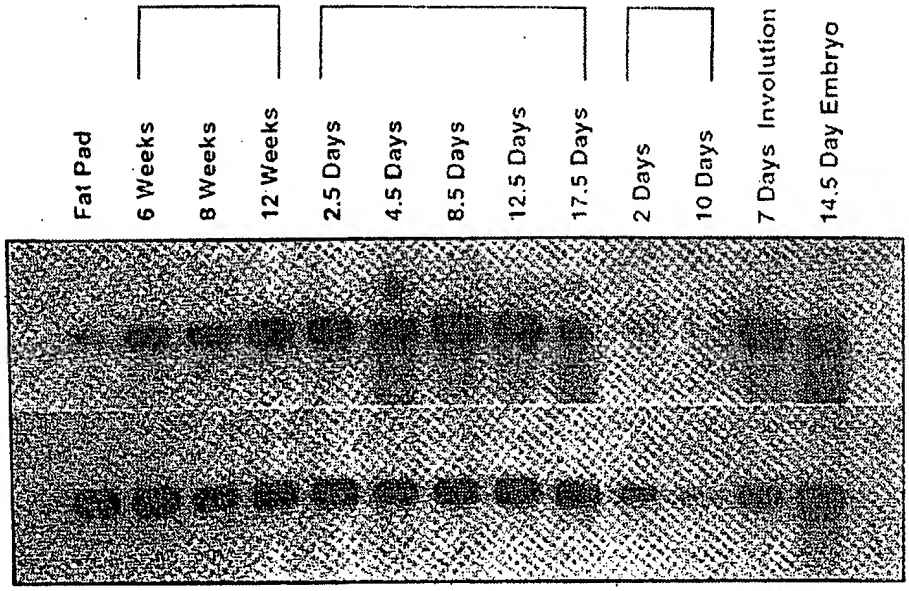

Virgin
Pregnancy
Lactation
Fat Pad
6 Weeks
8 Weeks
12 Weeks
2.5 Days
4.5 Days
8.5 Days
12.5 Days
17.5 Days
2 Days
10 Days
7 Days Involution
14.5 Day Embryo

FIG. 3

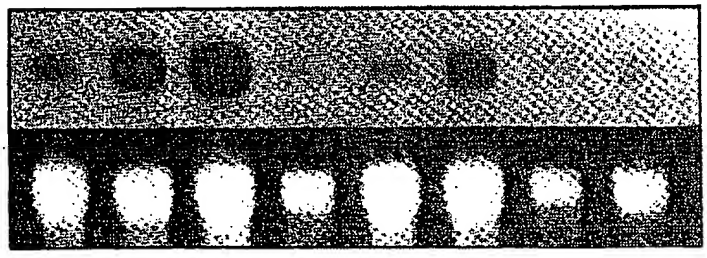

Normal Breast
Pregnant Breast
Carcinoma in Pregnant Breast
Grade III
Breast Carcinomas

FIG. 5

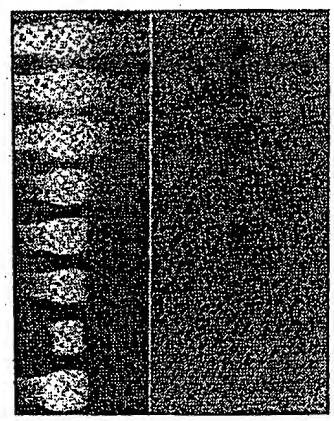


FIG. 6a

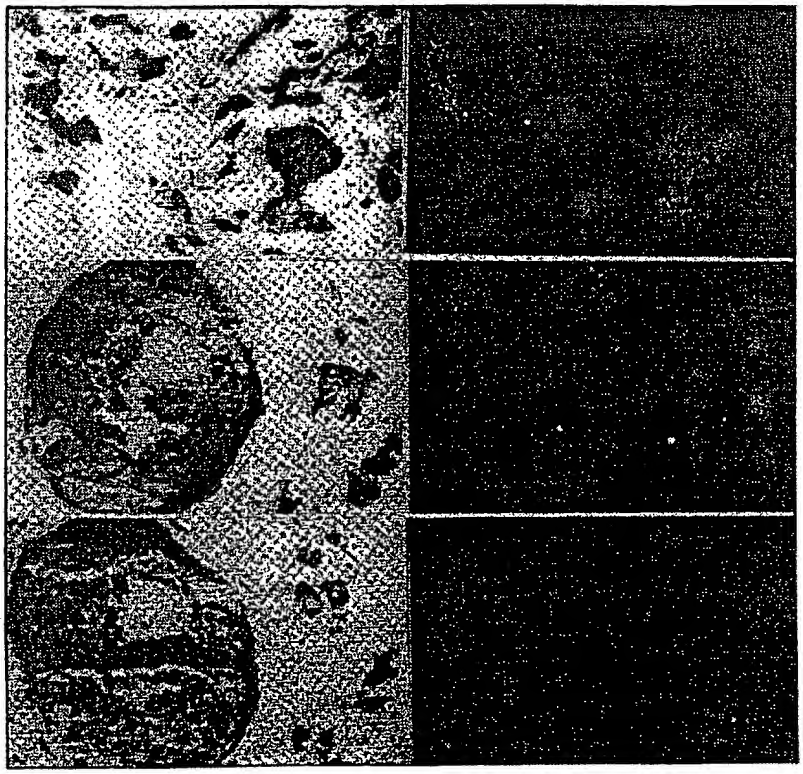


FIG. 6b

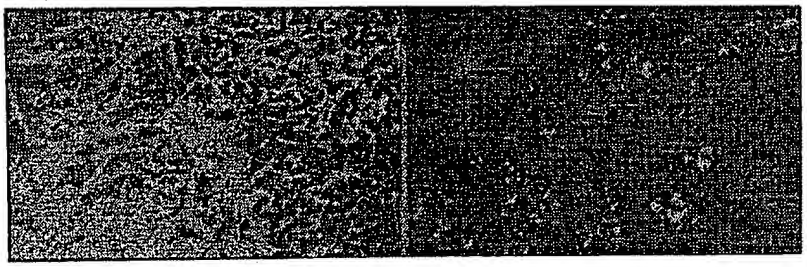


FIG. 6c

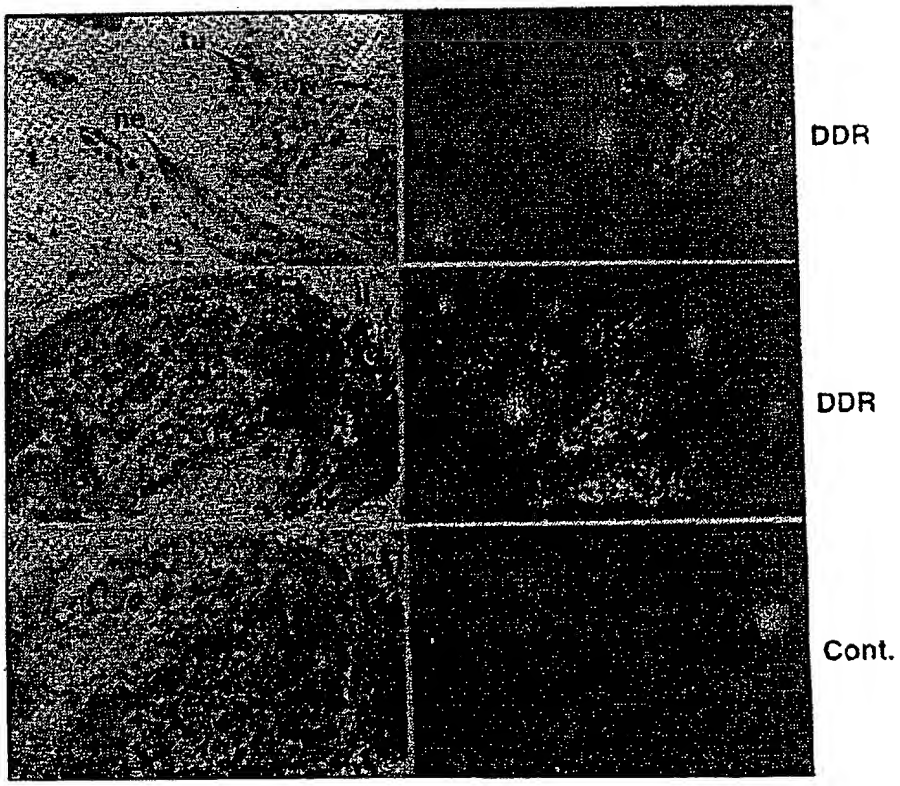
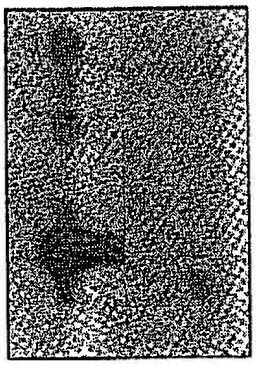
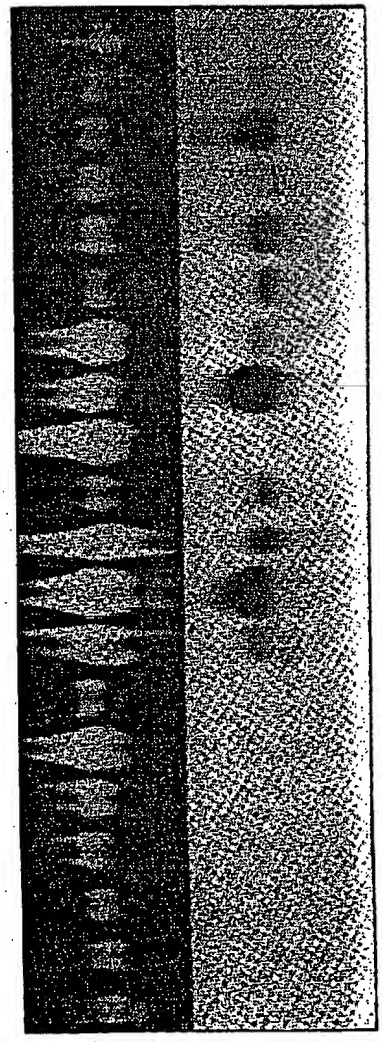


FIG.7a



Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas

FIG.7b



Oesophagus
Stomach
Gall Bladder
Ileum
Caecum
Colon
Tonsil
Thyroid
Spleen
Kidney Pelvis
Kidney Medulla
Kidney Cortex
Lung
Skin
Testis
Prostate
Myometrium
Endometrium
Breast
Bone Marrow

INTERNATIONAL SEARCH REPORT

Intern ual Application No
PCT/GB 94/01480

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/574 C12Q1/48 A61K31/55 A61K31/275 A61K31/35
A61K39/395 A61K38/00 A61K31/70 G01N33/573 C12Q1/68
C12N9/12 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.90, June 1993, WASHINGTON US pages 5677 - 5681 JEFFREY D. JOHNSON ET AL. 'A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain' cited in the application see the whole document ---	1-25
Y	DRUGS OF THE FUTURE, vol.17, no.2, 1992 pages 119 - 131 TERRENCE R. BURKE, JR. 'Protein-tyrosine kinase inhibitors' cited in the application see the whole document ---	1-25

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 1994	08.12.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Döpfer, K-P

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INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/GB 94/01480

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MEDICINAL CHEMISTRY, vol.34, 1991, WASHINGTON US pages 1896 - 1907 AVIV GAZIT ET AL. 'Tyrphostins. 2. Heterocyclic and alpha-substituted Benzylidenmalononitrile Tyrphostins as Potent Inhibitors of EGF Receptor and ErbB2/neu Tyrosine Kinases' cited in the application see the whole document ---	1-10, 19-21
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.89, May 1992, WASHINGTON US pages 4285 - 4289 PAUL CARTER ET AL. 'Humanization of an anti-p185(HER2) antibody for human cancer therapy' cited in the application see the whole document ---	1-7, 11-13, 15,19, 22-25
P,A	ONCOGENE, vol.8, no.12, December 1993, BASINGSTOKE GB pages 3433 - 3440 THOMAS KARN ET AL. 'Stzructure, expression and chromosomal mapping of TKT from man and mouse: a new subclass of receptor tyrosine kinases with a factor VIII-like domain' see the whole document ---	1-8, 11-19, 22-25
P,A	ONCOGENE, vol.9, no.1, January 1994, BASINGSTOKE GB pages 211 - 219 JOSE L. PEREZ ET AL. 'Identification and chromosomal mapping of a receptor tyrosine kinase with a putative phospholipid binding sequence inits ectodomain' see the whole document ---	1-8, 11-19, 22-25
P,A	ONCOGENE, vol.8, no.10, October 1993, BASINGSTOKE GB pages 2731 - 2739 MARIELBA ZERLIN ET AL. 'NEP: a novel receptor-like tyrosine kinase expressed in proliferating epithelia' cited in the application see the whole document ---	1-8, 11-19, 22-25
1 A	EP,A,0 504 914 (E.R. SQUIBB & SONS, INC.) 23 September 1992 see the whole document --- -/--	1-8, 11-19, 22-25

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/GB 94/01480

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 10234 (EURO-DIAGNOSTICS B.V.) 7 September 1990 see the whole document	1,4
A	SCIENCE, vol.241, no.4861, 1 July 1988, LANCASTER,PA, US pages 42 - 52 STEVEN H. HANKS,ANNE MARIE QUINN, TONY HUNTER 'The Protein Kinase Family: Conserved Features and Deduced Phylogeny of the Catalytic Domaines' cited in the application	
A	JOURNAL OF CLINICAL ONCOLOGY, vol.10, no.7, July 1992, NEW YORK, NY, US pages 1049 - 1056 B.A. GUSTERSON ET AL. 'Prognostic Importance of c-erbB-2-Expression in Breast Cancer' cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 94/01480

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0504914	23-09-92	JP-A-	5273208	22-10-93
WO-A-9010234	07-09-90	NL-A-	8900529	01-10-90
		AU-A-	5263190	26-09-90
		EP-A-	0461174	18-12-91
		JP-T-	4504171	23-07-92